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Quantitative assessment of mitochondrial morphology relevant for studies on cellular health and environmental toxicity

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1 1 Quantitative assessment of mitochondrial morphology relevant for studies on cellular health and
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3 2 environmental toxicity
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MITOMATIQUE

MITOCHONDRIA ENLIGHTEN

WORKFLOW

- Miniaturized
- Automated
- Integrated
- Multidimensional
- Multiscale

CONCEPT



APPLICATIONS

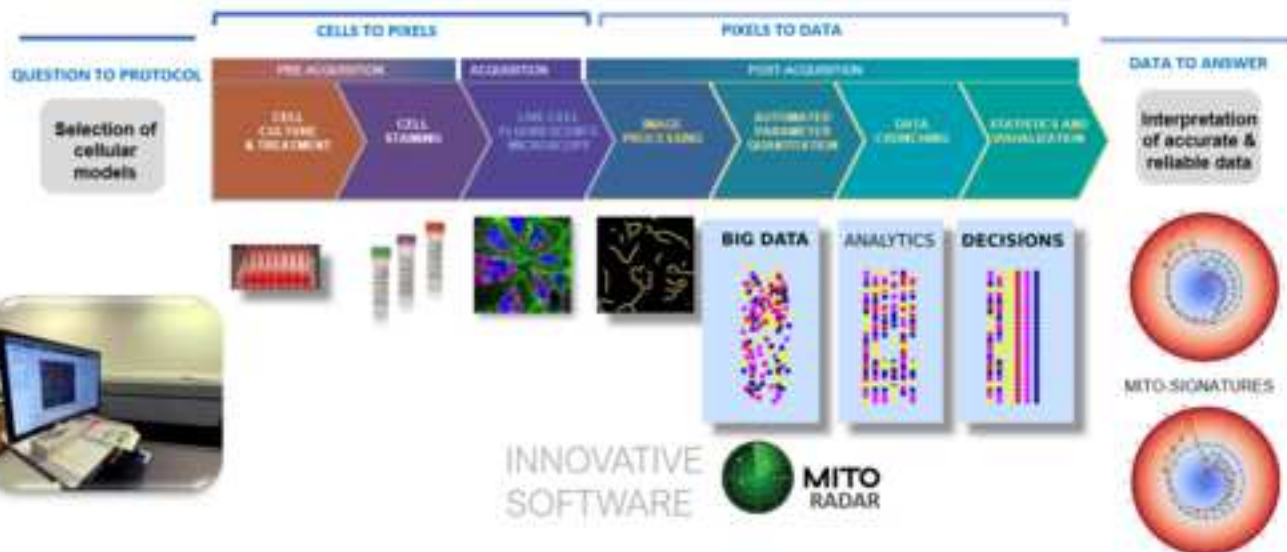
Toxicology
Predictive
Environmental

Health
Biomarkers
Screening

Cosmetics
Objectivation
Claim substantiation

EXAMPLE

Compared mitochondrio-toxicity (x5) of a cocktail of pesticides versus main pesticide



24 [Abstract \(250 words\)](#)

25 Mitochondria are essential organelles that play crucial roles in cellular energy metabolism, calcium
26 signaling and apoptosis. Their importance in tissue homeostasis and stress responses, combined to their
27 ability to transition between various structural and functional states, make them excellent organelles for
28 monitoring cellular health. Quantitative assessment of mitochondrial morphology can therefore provide
29 valuable insights into environmentally-induced cell damage.

30 High-content screening (HCS) provides a powerful tool for analyzing organelles and cellular
31 substructures. We developed a fully automated and miniaturized HCS wet-plus-dry pipeline
32 (MITOMATICS) exploiting mitochondrial morphology as a marker for monitoring cellular health or
33 damage. MITOMATICS uses an in-house, proprietary software (MitoRadar) to enable fast, exhaustive
34 and cost-effective analysis of mitochondrial morphology and its inherent diversity in live cells.

35 We applied our pipeline and big data analytics software to assess the mitotoxicity of selected chemicals,
36 using the mitochondrial uncoupler CCCP as an internal control. Six different pesticides (inhibiting
37 complexes I, II and III of the mitochondrial respiratory chain) were tested as individual compounds and
38 five other pesticides present locally in Occitanie (Southern France) were assessed in combination to
39 determine acute mitotoxicity. Our results show that the assayed pesticides exhibit specific signatures
40 when used as single compounds or chemical mixtures and that they function synergistically to impact
41 mitochondrial architecture.

42 Study of environment-induced mitochondrial damage has the potential to open new fields in
43 mechanistic toxicology, currently underexplored by regulatory toxicology and exposome research.
44 Such exploration could inform health policy guidelines and foster pharmacological intervention, water,
45 air and soil pollution control and food safety.

46 [Keywords](#)

47 Mitochondria; quantitative imaging; cellular stress ; confocal microscopy ; high content analysis; live-
48 cell imaging; environmental health; pesticides.

49 Short title

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50 Quantitative imaging and phenotyping of mitochondria for toxicity studies.

51

52 1. Introduction

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3 53 Mitochondria play a vital role in ATP production and in many critical cellular processes (1,2).
4
5 54 Mitochondrial markers are among the first to change upon homeostasis disruption, such as when cells
6
7 55 are exposed to environmental stress (3–5) (e.g. toxicants), and during disease (6,7) and aging (8). By
8
9 56 sensing and responding to changes in the cellular environment, mitochondria orchestrate adaptive
10
11 57 responses that extends well over cellular boundaries to impact tissue, organ and ultimately organism
12
13 58 physiology (9). The importance of mitochondria in tissue homeostasis, stress responses and diseases,
14
15 59 combined to their ability to transition between various structural and functional states, make them
16
17 60 excellent organelles for monitoring cell health (10).

20
21 61 Healthy mitochondria are usually mobile, tubular and interconnected, whereas cells under stress or
22
23 62 entering apoptosis often display swollen or fragmented mitochondria, marked by concurrent disruption
24
25 63 of metabolism and excess production of reactive oxygen species (ROS) (11). Mitochondrial change in
26
27 64 shape and size depending on their functional status, for example going through cycles of fission and
28
29 65 fusion to improve mitochondrial quality (12). Thus, mitochondrial morphological phenotypes are
30
31 66 complex and heterogeneous. The morphological diversity of this highly dynamic organelle varies from
32
33 67 small punctate structures to well-developed reticular networks with numerous intermediate states in-
34
35 68 between. To define or classify mitochondrial (network) architectures, only a few terms are usually used,
36
37 69 such as *short, long, fragmented, interconnected, tubular, ramified, compacted, aggregated, dislocated,*
38
39 70 *fissioned or hyperfused* (13–15). These terms are often used in an operator-dependent, subjective way.

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44 71 In recent years, to address the challenge of describing this inherent mitochondrial diversity more
45
46 72 objectively, several approaches which apply image processing algorithms and morphometric methods
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48 73 have been developed (16–18), providing valuable insights into tissue physiology, pathology and damage
49
50 74 (19–21). However, most of these programs are difficult to utilize in standard biology laboratories since
51
52 75 they require extensively trained staff and only handle the morphological computation steps, with no
53
54 76 visual display, statistical analysis or decision-making support. In most cases, only a limited number of
55
56 77 cells or conditions are analyzed with such software, creating ambiguity about the robustness of the
57
58 78 obtained data and ultimately as to the true utility of such software.

79 Over the last few years, we developed a sophisticated and innovative pipeline (MITOMATICS)
80 exploiting mitochondrial morphology as a marker for monitoring cellular health or damage.
81 MITOMATICS uses computerized methods to enable accurate, fast and cost-effective analysis of
82 mitochondrial shape and network architecture from confocal fluorescent images acquired from cultured
83 living cells. Three successive versions of this in-house, proprietary software were produced: MitoShape
84 (2 shape descriptors) (22), MitoTouch (31 descriptors) (Charrasse et al., submitted) and the here within
85 described MitoRadar (104 shape descriptors). This software is easy to use and does not require any
86 special skills in computer science or image processing. In contrast to previous versions, numerous
87 parameters have been added to provide a multiscale analysis of the mitochondrial component (from
88 solitary mitochondria to mitochondrial clusters present within single cells or in a cell population) and
89 the segmentation step is now performed by deep learning, enhancing image quality and enabling
90 accurate analysis. As a result, MitoRadar generates specific ‘mito-signatures’, some of which signal
91 cell safety whereas others may provide early predictors of cell danger. In the present study, we applied
92 MitoRadar in the context of predictive and environmental toxicology by assessing mitochondrial
93 architecture in live human cells exposed to specific chemicals, including pesticides used alone or in
94 combination.

96 2. Materials and methods

97 2.1.1. Chemicals

98 Dimethyl sulfoxide (DMSO; CAS number 67-68-5), Carbonyl cyanide m-chlorophenyl hydrazone
99 (CCCP; CAS number 555-60-2), Rotenone (CAS number 83-79-4), Antimycine A (CAS number 1397-
100 94-0), Mitochondrial Division Inhibitor MDIVI-1 (CAS number 338967-87-6), Mitochondrial Fusion
101 Promoter M1 (CAS number 219315-22-7), Fenpyroximate (CAS number 134098-61-6), Pyridaben
102 (CAS number 96489-71-3), Mepronil (CAS number 55814-41-0), Thifluzamide (CAS number 130000-
103 40-7), Azoxystrobin (CAS number 131860-33-8), Pyraclostrobin (CAS number 175013-18-0), Folpet
104 (CAS number 133-07-3), Pendimathaline, Chlorpyrifos-Methyl (CAS number 5598-13-0), Lindane

105 (CAS number 58-89-9), Cyprodinil (CAS number 121552-61-2) were purchased from Sigma Aldrich.
106 Stock solutions between 2 and 100 mM were made in DMSO and stored at -20°C until use. Treatment
107 solutions were prepared freshly for each experiment with a final concentration of DMSO below 0.5 %
108 (v/v) in culture medium.

2.1.2. Cell culture and treatments

109 A549 (CCL-185), RPE-1 (CRL-4000), U2OS (CRL-3455), Hs68 (CRL-1635), MDA-MB-231 (HTB-
110 26), THLE-3 (CRL-11233), HK-2 (CRL-2190) and BEAS-2B (CRL-9482) cell lines were purchased
111 from ATCC (American Type Culture Collection, LGC, Germany) and cultured in a 5% CO₂ atmosphere
112 at 37°C according to ATCC recommendations. To increase the susceptibility of cells to mitochondrial
113 toxicants, culture medium was replaced by freshly prepared glucose-free medium supplemented with
114 10mM galactose. For treatment, indicated concentrations of chemicals were added to the culture
115 medium. LD50 was determined by counting nuclei 24h after drug exposure. Four biological replicates
116 were performed for all experiments and each experiment was repeated at least 3 times. For the
117 *MitoCocktail* experiments, the most prominent pesticides were tested at concentrations from 10 to
118 500µM and solutions were prepared according to a rule of conservative ratios, i.e., by mixing different
119 pesticides with relative proportions (expressed in µM) based on the ATMO report (where pesticide
120 concentrations are expressed as ng of substance per m³ of air).

2.1.3. Cell staining and HCS Imaging

121 A total number of 20,000 to 40,000 cells were seeded onto 96-well black polystyrene microplates
122 (CLS3603, Corning®) 24 to 48h prior conducting experiments. Mitochondrial morphology was
123 examined after staining with 250 nM MitoTracker Deep Red FM (M22426) diluted in phenol red-free
124 culture medium in presence of 2.5 µg/mL Hoechst 33342 (H21492) for 30 min at 37°C. Cells were then
125 washed twice with PBS and labelled with 6.25 µg/mL CellMask Green Plasma Membrane Stain
126 (C37608) for 5 min at 37°C. All these vital dyes were purchased from Thermofisher Scientific (Life
127 Technologies SAS, Courtaboeuf, France). Mitochondrial network, nuclei and membranes of live cells
128 were imaged using the Opera Phenix® High-Content Screening System (PerkinElmer Inc.). Confocal
129

131 image acquisition (spinning disk) was performed using a 63x water immersion lens (1.15NA LD C-
132 Apochromat) and 5 fields were imaged per well. Automated image capture on a 16bit sCMOS camera
133 (pixel 6.5 μm) using 640 nm/488 nm/405 nm lasers in the indicated order, with Phenix emission filters
134 650-760 nm/500-550 nm/435-550 nm, has been optimized to provide high image quality and resolution.
135 In particular, laser power and exposure time were adjusted to maximize signal without saturation and
136 to minimize photobleaching and background noise (20% power, 100 ms for the far-red channel, 80%
137 power, 500 ms for the green channel and 100%, 200 ms for the blue channel).

2.1.4. Quantification of mitochondrial parameters using MitoRadar

139 As described below, the MitoRadar software (APP deposit number: IDDN.FR.001.470036
140 .000.S.P.2022.000.31235) consists of several modules designed for data handling, data processing,
141 detection and comprehensive analysis of mitochondrial changes in HCS imaging data.

2.1.5. Basic image import and processing

143 First, a loading module enables the import of fluorescence images from high-content imaging (HCI)
144 microscopes. A plate module can be used to add experimental data information during the loading stage.
145 A data handling module allows for the sorting of images and assignment of plate data, well data, and
146 experimental data. It can also be used to add and update experimental data information at any time
147 during the analysis process.

148 The segmentation module of MitoRadar uses deep learning and state-of-the-art image processing
149 techniques to detect cells, nuclei and mitochondria in HCS images (Fig. S1 A) at various scales (Fig.
150 S1 B). The MitoRadar segmentation technique utilizes a tri-channel fluorescence image input with
151 different colors associated to nuclei, mitochondria and cytoplasm. In this study, 2160x2160 images
152 were analyzed at a resolution of 94nm.pix-1 (Fig. S1 C panel a).

153 Next, both nucleus and cell channels undergo a normalization process between 0 and 1 utilizing a
154 quantile normalization approach between quantile 1% and quantile 99%. A trained convolutional
155 network (Cellpose uNet) (23,24) is applied to each channel to perform instance segmentation of cell
156 and nuclei. The uNet produces a probability map and a gradient flow which enables the reconstruction

157 of object instances. This method was proven to be effective for cell segmentation of objects with
158 irregular shapes (25–27). To reduce computation time, the images are down-sampled to 224 pixels
159 before entering the neural network and are up-sampled post-analysis using nearest neighbors followed
160 by a median filtering technique to smooth edges (Fig. S1 C panel b). The nuclei instances are then
161 associated with cells utilizing a 1-to-1 association rule, with the greater intersection size used to resolve
162 conflicts when necessary (Fig. S1 C panel c).

163 Due to variations in mitochondrial intensity between cells within the same field, mitochondrial
164 segmentation is performed cell by cell following the segmentation of nuclei and cells. The
165 corresponding bounding box within the mitochondrial channel is extracted for each cell, and the average
166 intensity inside the cell mask is utilized for background noise removal. The intensities are normalized
167 between 0 and 1 utilizing a quantile normalization approach between quantile 0% and quantile 99.9%.
168 Next, a Gaussian difference threshold ($\sigma=0.9 \mu\text{m}$, $t=0.05$) and a Laplacian of Gaussian threshold
169 ($\sigma=0.9 \mu\text{m}$, $t=0$) are applied to produce a mitochondrial mask for the cell. Clusters are defined as
170 connected components on this mask (Fig. S1 C panel d) and are refined by removing small objects (size
171 < 16 pixels or $0.13 \mu\text{m}^2$) followed by morphological closing. The skeletons of mitochondrial clusters
172 are extracted utilizing Zhang's algorithm (28) (Fig. S1 C panel e). Finally, to detect each individual
173 instance of mitochondria, a watershed approach is applied to mitochondrial intensities using the
174 skeleton branches as seeds and the mitochondrial mask as the mask (Fig. S1 C panel f). Moreover, this
175 segmentation module can be parallelized and utilizes the GPU for fast processing of a large number of
176 images.

2.1.6. Automated parameter quantification with integrated statistics

178 After segmentation, pictures are declumped and a quantification module then collects 104
179 morphological, intensity and texture parameters (hereby referred to as 'morphological descriptors'; see
180 Table S1 for the exhaustive list and further details) on cells, nuclei, mitochondria and mitochondrial
181 clusters. These descriptors can be used to characterize various aspects of mitochondrial architecture,
182 such as size, shape, density and organization within the cellular area. Note that the current version of
183 the software does not normalize descriptor values based on the cell size. Finally, the analysis module

184 of MitoRadar allows users to make state-of-the-art statistical tests and plots on the resulting
185 quantifications and to easily compare experimental conditions. This module also includes integrated
186 linear discriminant analysis (LDA) and principal component analysis (PCA) for exploring multivariate
187 effects and a specific MitoRadar plot to access both significance and amplitude information.

188 The MitoRadar analysis module can calculate a MitoScore based on a cross validation of the impact
189 (difference with the basal condition) and number of all affected parameters. First, a hierarchical
190 clustering is applied to remove redundancies among descriptors. Then, 20 LDA models are trained on
191 50% fold random samples of the data to differentiate between the control and the trial conditions. These
192 are then tested on the complementary folds using the average balanced accuracy between all LDA
193 models as a score showing how easily a ML algorithm can discriminate between control and trial. The
194 top 15 significant uncorrelated descriptors that discriminate the compared conditions are then shown in
195 a MitoRadar plot and a slider is placed along a heat color scale, leading to the delineation of five distinct
196 categories (*No effect, Limited / Substantial / Measurable or Large overall effect*) (Fig.1).

198 3. Results

199 3.1.1. Design of the MITOMATICS workflow

200 Quantitative imaging of cellular structures and substructures like mitochondria requires rethinking low-
201 throughput experimental procedures to include the use of multi-well plates (rather than tissue culture
202 petri dishes or small imaging chambers), live cell techniques (instead of fixed-cell imaging methods),
203 random imaging of multiple fields (rather than operator-dependent monitoring of only one chosen
204 microscope field encompassing multiple cells), databases of high-quality, classified images (rather than
205 production of a limited number of acquired images), automated digital image processing (instead of
206 manual counting) and rapid, massive data gathering, analysis and visualization (rather than slow
207 performance data analysis with suboptimal charts, graphs or histograms hindering easy and correct data
208 interpretation). The MITOMATICS wet-plus-dry pipeline has been specifically designed for high-
209 throughput phenotyping of mitochondrial morphology by miniaturizing and automating high-resolution

210 imaging before an in-house software application (MitoRadar) handles all fundamental operations from
211 image segmentation to statistical analysis and drawing meaningful interpretation from custom mito-
212 signatures (Fig. 1). The use of 96-well plates with random imaging of 5 fields per well enables analysis
213 of multiple experimental conditions while achieving robust results. To image mitochondria in their
214 cellular context, we use the potentiometric dye MitoTracker Deep Red (MTDR) in combination with
215 fluorescent markers (green and blue, respectively) specific for cellular membranes (Cell Mask Green)
216 and nuclei (Hoechst 33342). Confocal images are acquired on an Opera Phenix® High-Content
217 Screening (HCS) System (equipped in its optimal configuration with a robotic arm and incubator) and
218 directly loaded into the MitoRadar software to perform the post-acquisition steps. This software uses
219 deep learning during the image segmentation phase (Fig. S1) and calculates about a hundred
220 morphological descriptors (Table S1), offering one of the most comprehensive sets of parameters to
221 infer mitochondrial (network) shape and overall cell morphology. The MitoRadar software enables the
222 detection and assessment of subtle morphological characteristics, undetectable or barely detectable
223 through conventional methods and has advanced analytical, statistical and graphical features for data
224 representation, analysis and interpretation. Our miniaturized technology suits diverse applications
225 among which are: testing the noxious effect of individual molecules on the mitochondrial network
226 (*MitoCollapse* module for predictive toxicology) ; testing the deleterious effects of combinations of
227 molecules, drugs or pollutants (*MitoCocktail* module for predictive as well as environmental
228 toxicology) ; assessing changes in mitochondrial morphology in pathological (or aged) cells compared
229 to their non-pathological (or ‘young’) counterparts (*MitoMedCare* module) and objectifying the
230 beneficial effects of cosmetic or pharmacological bioactive ingredients (*MitOasis* module) (Fig. 1). In
231 the present report, we focus only on the first two applications.

3.1.2. Implementation of the MitoRadar software

234 Here, we sought to validate our fully automated image analysis pipeline on rich datasets gathered from
235 cultured live human cells exposed to specific chemicals known to induce either mitochondrial
236 fragmentation (CCCP, Rot, Ant A) (29–32) or mitochondrial hyperfusion (MDIVI-1, M1) (33,34).

237 Eight human cell lines of normal and diseased states, from different tissue origin, were successfully
238 imaged (Fig. S2), leading to an image database of approximately 30,000 microphotographs (named
239 ‘MITOPIX-DEV’, DEV standing for ‘development’) that was used to develop the MitoRadar software.
240 Representative images are shown in Fig. 2 for BEAS-2B cells (see Fig. S3 for the other cell lines), a
241 human lung epithelial cell line that has been widely used in toxicity tests, specifically to assess the
242 deleterious effects of heavy metals (35), microplastics (36), fine particles (37) and pesticides (38).

243 Based on MITOPIX-DEV, the MitoRadar software was implemented, optimized and extensively
244 debugged over a six-month period. Several functionalities were implemented: (i) an AI image
245 segmentation module (using deep learning) to detect cells, nuclei and mitochondria (Fig 3A, 3B panel
246 a and Fig. S1 A); (ii) a quantification module to characterize the detected objects with advanced
247 descriptors (n=104, see Table S1 for the exhaustive list); note that for each descriptor, 4 distribution
248 aggregators were computed: mean, variance, skewness and kurtosis, allowing for a more refined and
249 realistic description of the objects under study; (iii) a database with its dedicated interface dynamically
250 linking the data and metadata of the experiments (Fig. 3B panel b); and finally (iv) an interface for
251 integrated analysis (including statistics) and easy visualization of the results (Fig. 3B panels c-d and
252 Fig. 3C).

253 MitoRadar provides a comprehensive analysis of the morphological characteristics of the
254 mitochondriome of cells by performing morphometric measurements at the supra-mitochondrial,
255 mitochondrial and sub-mitochondrial levels. The computer program is multiscale, allowing to navigate
256 over a diverse range of scales (isolated mitochondria, clusters of mitochondria, subcellular locations,
257 cells, cell cultures) (Fig. S1B) and multidimensional, multivariate quantitative data being readily
258 visualizable through innovative radar charts. Each variable is represented along the X-axis. The
259 computed parameters are compared to a reference situation (blue circle) and parameters showing
260 statistically significant deviation are displayed along a heat map Y-axis (the size of the points along the
261 curve reflecting the degree of statistical significance). Six different MitoRadar plots can be created by
262 grouping the parameters associated with single or networked mitochondria, the total mitochondrial
263 complement present in a given cell (mitochondriome), cellular features, nuclear features and attributes

264 of the *counter shape* (i.e., inverted selection of the cellular region containing labelled mitochondria, to
1 our best knowledge this latter category of descriptors being completely new in the field) (see Fig. 3C).
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4 266 Parameters that show little or no variation compared to the control are listed in brown, those that
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6 267 decrease or increase are respectively colored in blue and red, with fixed rank ordering or (according to
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9 268 user preference) with a rank assigned in descending or ascending order.

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11 269 Results are generated in the form of reports of decreasing complexity, integrating statistical analysis
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13 270 and advanced graphical representation tools (two functionalities absent from most image analysis
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15 271 software, which require separate manual processing by a trained operator). The colors and their
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18 272 intensities are correlated with the size of the measured effects, and statistical significance is represented
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20 273 by circles of increasing diameter (Fig. 3C). The user can choose between PCA, LDA, correlation map
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22 274 or dendrogram, T-Test with corrections for multiple comparisons, and SSMD (Fig. 3B panels c-d). All
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24
25 275 of these advanced statistical tests are natively integrated into MitoRadar's user-friendly graphical
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27 276 interface, which makes them accessible without any third-party software to biologists who do not have
28
29 277 programming skills. It also makes it easy to share projects, images, and quantifications.
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35 279 *3.1.3.MitoRadar validation on high-throughput microscopy images of cells treated with the* 36 37 280 *mitochondrial uncoupler CCCP*

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40 281 During cellular stress when ROS accumulates the mitochondrial network becomes largely fragmented
41
42 282 (39). CCCP, an inhibitor of mitochondrial oxidative phosphorylation, evokes the integrated stress
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44 283 response and its application to cells results in dramatic fragmentation of mitochondria (29,30). To test
45
46 284 the performance of MitoRadar as a big data analytics software, we treated BEAS-2B cells with 20μM
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48 285 CCCP during 4h (Fig. 4A). Five independent experiments were done with more than 200,000 cells
49
50 286 analyzed, representing about 32,580 microphotographs and a total processing time of around 260
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53 287 minutes (for the whole project). The three upper MitoRadar plots of Fig. 4B show a massive and highly
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55 288 significant variation of more than 90% of the descriptors (55/61) related to the mitochondrial phenotype,
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58 289 most of them (47) with $p < 0.001$ *** (Mann Whitney Test with Benjamini Hochberg correction). Among
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290 the descriptors that decrease (in blue) and account for size reduction of both mitochondria and
291 mitochondrial (sub)networks, we find `area_mean`, `aspect_ratio_mean`, `axis_major_length_mean` and
292 `axis_minor_length_mean`, `equivalent_diameter_area_mean`, `feret_diameter_max_mean` and
293 `perimeter_crofton_mean`.

294 Mitochondrial fragmentation is evidenced by the augmentation (in red) of circularity and roundness.
295 Smaller degrees of mitochondrial interconnection and branching are evidenced by the decrease in
296 skeleton length and number of branch points (`skel_length_mean`, `branch_points_mean`,
297 `clusterised_mito` and `clustering_coef`). Consistent with this dislocation of the mitochondrial reticulum,
298 the number of mitochondria and specifically of isolated mitochondria appears greater. Texture as well
299 as density profiles undergo concomitant variations as indicated by increase in `solidity_mean` and in
300 Euler number means, which relate to the number of holes in the analyzed cells when mitochondria and
301 mitochondrial clusters are considered (Fig. 4B, top right panels) and in their inferred negative shapes
302 or *countershape* (Fig. 4B, bottom middle panel). Subcellular distribution of the mitochondria also
303 reflects cellular stress as shown by their positioning away from the cell membrane and closer to the
304 nucleus.

305 Compared to mitochondrial parameters, changes in cellular parameters are less drastic (Fig. 4B, bottom
306 right plot) and suggest slight spreading of the cells. Because the set of nuclear parameters (n=18) does
307 not display any significant change at all (Fig. 4B, bottom left plot), our data demonstrate that MitoRadar
308 objectively measures mitochondria-damaging effects before the onset of early apoptotic features (such
309 as the appearance of highly condensed pyknotic nuclei, cell shrinking and blebbing). These
310 mitochondria-damaging effects are described as ‘large’ by an in-house scoring system, MitoScore (Fig.
311 4C), which comprises a color-coded scale and a summary of the most affected parameters (see *Methods*
312 for details).

313 MitoRadar software natively offers a range of both basic and fairly advanced statistical tools for data
314 analysis, such as PCA, LDA and violin plots (i.e., hybrids of box plots and kernel density plots) (Fig.
315 4D). Details on SSMD values (Fig. S4 A), u-values (Fig. S4 B) and p-values (Fig. S4 C) were also
316 plotted separately for better clarity (readability) and counting of u-values. Lastly, note that

317 mitochondrial descriptors show a high level of correlation on both correlation dendrogram (or
318 hierarchical clustering diagram) (Fig. S4 D) and distance map (Fig. S4 E), revealing a global
319 transformation of the mitochondrial component. The PCA plot shows that the control and CCCP
320 conditions clearly segregate into two distinct groups (Fig. 4D, panel a). Projection of the descriptors
321 onto the axes of the PCA confirms that the observed variations are mainly due to changes in
322 mitochondrial features (Fig. S4 F). LDA with prior knowledge of class labels improves the observed
323 segregation between the control and CCCP-treated groups (Fig. 4D, panel b and Fig. S4 G). Violin plots
324 are useful for plotting variations in individual descriptors as exemplified here by the Mito
325 roundness_mean variable (Fig. 4D, panel c). Altogether, our results suggest that MitoRadar operates
326 quickly, efficiently, reliably and cost-effectively to distinguish a highly fragmented mitochondrial
327 network from a normal, healthy one using live-cell fluorescence microscopy images of cultured
328 mammalian cells.

3.1.4. *MitoCollapse: assessing acute mitochondrio-toxicity of single pesticides in vitro*

331 Exposure to anthropogenic pollutants is a critical biomedical and ecological issue, pesticides being
332 potentially hazardous to human health and one of the main contributors to environmental deterioration.
333 We hypothesized that our approach could be useful to assess the in vitro mitochondrial toxicity of a
334 selection of pesticides, taking CCCP as an internal positive control of cell poisoning. Four different
335 fungicides were chosen, including the two ETC complex II inhibitors Mepronil (MEP) and
336 Thifluzamide (THI) as well as Azoxystrobin (AZO) and Pyraclostrobin (PYRA), which are two ETC
337 complex III inhibitors, plus the two acaricides Fenpyroximate (FEN) and Pyridaben (PYRI), which are
338 ETC complex I inhibitors.

339 Pulmonary BEAS-2B cells were treated with various concentrations (10, 50, 100, 250 and 500 μ M) of
340 individual pesticides and images were taken 2h after pesticide exposure. The threshold dose (i.e., the
341 dose below which MitoRadar plots show no variation compared to control) and LD50 (i.e, the dose for
342 which 50% of cells died 24h after) were then evaluated (Fig 5A). MitoRadar analysis corresponding to

343 features obtained with the 250 μ M dose (Fig. 5B) showed a clear disruption of mitochondria and their
344 networks in cells following single pesticide treatment, compared to control cells, resulting into a
345 MitoScore transitioning from ‘*Substantial overall effect*’ to ‘*Large overall effect*’ (Fig. 5C).

346 Like CCCP, all of these pesticides appeared to trigger mitochondrial fragmentation when applied at a
347 dose of 250 μ M, MitoRadar plots showing an increase in the circularity_mean, roundness_mean, and
348 isolated_mito parameters with concomitant decrease in skel_length_mean, area_mean,
349 axis_major_length_mean and axis_minor_length_mean.

350 Interestingly, analysis with MitoRadar also revealed several unexpected differences between the
351 measured effects of the various pesticides. For example, average length of the minor axis of
352 mitochondria decreased after treatment with CCCP, THI and PYRA, while a significant increase was
353 observed for this parameter with the other pesticides. Likewise, the number of mitochondria was
354 increased for CCCP as well MET and THI (inhibitors of complex II) and decreased for AZO and PYRA
355 (inhibitors of complex III). When LDA was carried out on defined pairs of pesticides according to their
356 type of ETC inhibition, untreated controls were well separated from the defined groups (as well as from
357 the CCCP-treated group) by the first two discriminant axis (Fig. 5D).

358 Compared to the negative control (vehicle), the Mito euler_number_mean, which reflects the number
359 of holes in the mitochondrial network, was smaller for the group of Complex III inhibitors, followed by
360 Complex II and I inhibitors, while skeleton length values progressively decreased with ETC inhibitors
361 I, III and II.

362 Nuclear parameters related to size tended to increase for CCCP while they decreased significantly for
363 PYRA. Few variations of the cell and countershape features were detected as shown on the MitoRadar
364 plots (Fig. S5 A). Details of the statistical analyses are presented in Fig. S5 (correlation distance MAP
365 Fig. S5 B; SSMD summary Fig. S5 C; U-Value Fig. S5 D).

366 Therefore, although as a first approximation the mitochondrial response to the tested single toxicants
367 was assumed to be similar (with pesticide treatment resulting in mitochondrial fragmentation), using

368 built-in statistical analysis modules, MitoRadar analysis was able to reveal subtle changes in the deep
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2 369 architecture of mitochondrial structures.

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6 7 8 371 *3.1.5.MitoCocktail: assessing acute mitochondrio-toxicity of pesticide mixtures in vitro*

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10 372 We took advantage of MITOMATICS for assessing the mitochondrial toxicity of pesticides
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12 373 administered in combination on cultured BEAS-2B cells, according to real-world data (40) produced
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14 374 by ATMO-Occitanie, the accredited association for air quality monitoring in the Occitanie Region
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16 375 (France). Annual campaigns to measure pesticides in the ambient air have been carried out over 12
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18 376 months since 2014 in a rural environment dominated by wine, arboriculture or field crops. Based on the
19
20 377 cumulative concentrations of the fifteen most commonly found pesticide residues in the different
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22 378 departments of Occitanie (Tarn-et-Garonne, Pyrénées-Orientales, Lauragais, Aude and Gard), the top
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24 379 five air-borne pesticides were selected for further analysis. The list of pesticides under study comprised
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26 380 two fungicides, Folpel (FOL) and Cyprodinil (CYP), one herbicide, Pendimethaline (PEN) and two
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28 381 insecticides, Lindane (LIN, banned since 1998) and Chlorpyriphos methyl (CHL) (Fig. 6A).

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31 382 First, these five pesticides were tested separately (as above) on BEAS-2B cells for 4h at various
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33 383 concentrations ranging from 10 to 500 μ M to determine the threshold dose. After 24h, the number of
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35 384 cells was recorded in each condition and the LD50 was calculated. A summary of the results from 4
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37 385 independent experiments (representing about 100,000 analyzed cells) is presented in Fig. 6A.

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40 386 Next, to determine if the pesticides can be more noxious when combined (a phenomenon known as the
41
42 387 ‘cocktail effect’), we prepared mixtures with relative proportions of pesticides based on the ATMO
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44 388 report. Note that the identity of the most prominent pesticide (which was tested at concentrations
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46 389 ranging from 10 to 500 μ M) varied across departments, representing between 78% and 97% of the final
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48 390 mixture (Fig. 6B).

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50 391 Our data indicate that both the threshold dose and the acute LD50 of pesticide mixtures were at least
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52 392 five times lower than that of individual pesticides in all considered departments (Fig. 6A and B). The
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54 393 case of Aude is taken here as an illustration. In this department, FOL is the main air-borne pesticide

394 (97%), whereas the three other pesticides (PEN, CHL and LIN) are present as traces (in the following
1 proportions: 1.1%, 0.8% and 1.5%, respectively).
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396 Mitochondrial disorganization was observable microscopically (Fig. 6C) and measurable through
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7 397 MitoRadar analysis (Fig. 6D) when 10 μ M FOL was mixed with low concentrations of the other
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10 398 pesticides (0.11 μ M PEN; 0.15 μ M CHL and 0.08 μ M LIN), i.e., when each individual pesticide was
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12 399 present at levels below its “no-observed-effect-concentration” (threshold doses were 50 μ M for FOL;
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14 400 >500 μ M for PEN; 100 μ M for CHL and 500 μ M for LIN).
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16
17 401 The computed MitoScore (based on the top 15 most affected parameters) indicated weak to moderate
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19 402 effects when cells were exposed to single pesticides at a 10 μ M concentration and maximal noxious
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21 403 effect with the pesticide mixture (Fig. 6E). In conclusion, traces of PEN, CHL and LIN potentiate both
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23 404 the mitochondrial and cellular toxicity of FOL. Note that similar conclusions can be drawn for the
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25 405 pesticide combinations characteristic of the other four departments.
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31 407 4. Discussion 32 33

34 408 Mitochondria are membrane-enclosed organelles ubiquitously found in eukaryotes. Originally derived
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36 409 from endosymbiotic bacteria, they play a vital role in energy production and in many other cellular
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38 410 functions (2,41). It is considered that all physicochemical, parasitic and microbial influences that
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40 411 surround eukaryotic life are translated into changes in mitochondrial structure and function (42–47). In
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42 412 particular, a growing body of literature points to mitochondria as a key organelle targeted by
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44 413 environmental pollutants (3–5,48–53). Not only do these environmental pollutants (present in air, water
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46 414 and soil) disturb the mitochondrial machinery but their mitotoxicity may cause significant damage to
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48 415 the epigenome and transgenerational inheritance of dysfunctional mitochondria (4). Mitochondria are
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50 416 thus considered to provide the missing link between cellular, organismal and environmental health (10).
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55 417 As ‘mitochondrial form follows function’ (54), previous studies highlighted the utility of using
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57 418 mitochondrial morphology as a proxy for monitoring cell health and cytotoxicity (17,46,55–59). In this
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59 419 paper, we described a comprehensive and user-friendly big data analytics software (MitoRadar)
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420 combined with a HCS wet pipeline (MITOMATICS) to analyze mitochondrial shape changes occurring
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2 421 in live cultured cells. We applied our image-based phenotypic profiling system to assess mitochondrial
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4 422 architecture in response to environmental pollutants. This system was designed to replace traditional,
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6 423 costly and low-throughput techniques, which will be reserved for downstream validation on prioritized
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8 424 combinations.

10
11 425 Because mitochondrial changes appear to happen before other cellular events, shorter incubation
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13 426 durations are needed, this approach thus saves time and money while enhancing efficiency. While most
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15 427 available tools require programming experience or computer science skills, the core component of our
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17 428 workflow involves an easy-to-work-with and intuitive in-house, proprietary software (MitoRadar).
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19 429 Live-cell, high-resolution confocal images are collected on any multiplexed imaging platforms
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21 430 available and from any type of cells cultured on 96-well plates and stained with different vital dyes
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23 431 (with one of them labelling mitochondria), before processing of a large number of images at various
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25 432 possible scales, AI-driven segmentation and automatic calculation by MitoRadar of 2D descriptors
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27 433 associated with the identified mitochondria in their cellular context.

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32 434 By quickly computing more than one hundred descriptors related to mitochondria (some of which being
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34 435 novel like the '*Countershape*') and additional nuclear and cellular shape parameters, MitoRadar
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36 436 represents one of the most comprehensive software packages available for image analysis. Our
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38 437 automated and miniaturized approach integrates a convenient way for interpreting the gathered massive
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40 438 and multidimensional data by creating unique radar plots in which sample datasets (e.g. from cells
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42 439 treated with chemicals) are compared to a reference dataset (i.e., untreated or mock-treated cells). These
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44 440 information-rich representations natively come up with a variety of statistical charts and plots (t-tests,
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46 441 PCA, LDA, violin distributions) and with a simple score (MitoScore), making it **quick and** easy to
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48 442 compare the impact of various substances or culture conditions and to detect subtle morpho-phenotypic
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50 443 variations. It is remarkable that our method was able to discriminate the effect of molecules acting at
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52 444 different stages of mitochondrial function, namely CCCP (a widely used mitochondrial uncoupler),
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54 445 Rotenone (Complex I inhibitor) and Antimycin (Complex III inhibitor). Besides CCCP and other
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56 446 traditional uncouplers (like FCCP or 2,4-dinitrophenol), novel mitochondria-specific uncoupling agents
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1 447 such as BAM15 and FR58P1 (60) or the organic pollutant pentachlorophenol (61) were identified that
2 448 could be tested using our technology to improve our knowledge of the consequences of mitochondrial
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4 449 uncoupling on mitochondrial network architecture. In addition to Antimycin A, which is specific to the
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6 450 N-side quinone binding site of Complex III, it would be interesting to assess the effects of Myxothiazol,
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8 451 which acts at the P-side. Inhibitors of Complex IV (potassium cyanide, sodium azide or lipophilic small
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10 452 molecules like steroids) and ATP synthase inhibitors like oligomycin and dicyclohexylcarbodiimide
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12 453 (DCCD), which respectively bind to the F₀ and F₀F₁ subunits of the proton pump, are also worth testing.
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16 454 Here, we applied our technology to test the noxious potential of single pollutants (*MitoCollapse*) as well
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18 455 as combination effects between molecules (*MitoCocktail*).
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21 456 Regarding *MitoCollapse*, we recently collected results on single exposure of skin cells to three
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23 457 pesticides (two insecticides, fipronil and imidacloprid as well as glyphosate, present in the widely used
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25 458 herbicide Roundup®) (Charrasse et al. submitted) and, in the present study, of non-tumorigenic lung
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27 459 cells to eleven additional pesticides including five currently detected in Occitanie (Southern France).
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30 460 This research is in line with classic toxicology studies carried out by various organizations such as the
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32 461 National Institute of Environmental Health Sciences through its National Toxicology Program (NTP).
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34 462 Interestingly, the mitochondrial ‘morpho-signatures’ of the six chemicals that we tested so far as
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36 463 individual substances appear not only to be distinct but also to segregate into discrete families,
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39 464 suggesting that the effects of pesticides (and virtually of any environmental pollutant(s)) might be
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41 465 classified within a ‘morphospace’ of mitochondriome morphologies. It would be interesting to
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43 466 determine whether specific signatures are linked to particular physicochemical properties of pesticides
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45 467 or to their toxicological profile.
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48 468 The combined effects of chemical mixtures, a rapidly emerging topic in environmental toxicology (62),
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50 469 prompted us to design another module, *MitoCocktail*, for estimating the mitochondrial response of a
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52 470 larger number of substances, across a battery of concentrations and in combination. This functionality
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54 471 currently handles projects using a single dose response of combined drugs, rather than a full two-way
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56 472 matrix of varying doses, similar to other HCS studies. Mixture effects are usually classified as being
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58 473 antagonistic, additive or synergistic, depending on whether the observed toxicity of the combination is
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1 474 lower than, equal to, or higher than the expected toxicity based on an additivity model. Thanks to
2 475 pollutant data made available by ATMO-Occitanie, we showed that the deleterious effects on
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4 476 mitochondrial architecture and cellular viability of the main pesticide found in each department of
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6 477 Occitanie were enhanced when trace quantities of other pesticides were added, indicative of
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8 478 synergistic relationships.

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11 479 MitoRadar analysis can help decipher human health impacts of anthropogenic chemicals found in the
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13 480 air, but also in water, food and soil. In addition to pesticides, various sources of pollution can be studied,
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15 481 such as fine particles, polycyclic aromatic hydrocarbons, plastics, pharmaceutical waste, cosmetics and
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17 482 even antibiotics (63) or noise pollution (64). Samples taken from natural environments and containing
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19 483 unidentified pollutants or metabolites may also be blind tested. Beyond exposure to pollutants, other
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21 484 variables may be considered such as genetic or epigenetic contexts, age, disease, diet and physical
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23 485 exercise that are likely to influence mitochondrial health. Our method is also suitable for analyzing
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25 486 primary cells from different organs or tissues of patients with particular life histories, such as for
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27 487 instance type 2 diabetes (65) or autism spectrum disorder (66), in order to gain better understanding of
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29 488 the mitochondrial correlates of pathophysiology. In that respect, it would be of interest to assay the
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31 489 morphometric parameters of mitochondria in cell lines derived from children with autistic disorder (in
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33 490 comparison to paired cells lines from typically developing siblings or control cell lines) with or without
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35 491 exposure to environmental factors such as air pollution or pesticides (67–70). This is indeed very
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37 492 relevant to this population as studies have shown changes in mitochondrial respiration with exposure to
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39 493 toxicants such as air pollution (69) and other common toxicants (71) in children with autism. This
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41 494 technique could be applied to disease populations to understand how environmental agents may produce
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43 495 disease through effects on bioenergetics. Taking up the One Health perspective (72,73), non-
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45 496 mammalian cell lines may also be tested including honeybee or zebrafish cell lines. Indeed, several
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47 497 pesticides (e.g. succinate dehydrogenase inhibitors, SDHIs) that are toxic to mitochondria act by
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49 498 blocking cellular respiration. This property, used to eliminate certain fungi, mites or worms, makes
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51 499 them potentially toxic for all living beings.

1 500 The MITOMATICS technology can be combined with phenotypic/functional analysis (e.g.
2 501 measurement of mitochondrial activities through ELISA plate readers and spectrophotometric methods,
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4 502 determination of apoptosis/necrosis rates by flow cytometry) and followed by more specialized readouts
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6 503 including measuring oxygen consumption and extracellular acidification rates (using Seahorse®),
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8 504 mitochondrial iron content through biochemical techniques or the uptake of specific fluorescent probes
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11 505 (like DCFH-DA for detecting ROS) by flow cytometry or fluorescent imaging. For example, in a recent
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13 506 study (66), mitochondrial morphology paralleled variations in respiratory rates in fibroblasts derived
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15 507 from patients and healthy controls, thereby allowing the validation of the respiratory changes and a
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17 508 better understanding of the consequence of these changes in mitochondrial respiration.

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20 509 Our approach may be improved in a number of ways. In particular, the mitochondriome should be
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22 510 viewed as a four-dimensional architecture ($X,Y,Z + \text{time}$) whose configuration may evolve due to
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24 511 mitochondrial fission, fusion and motility as well as cell movements (including various extension of the
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26 512 cell surface, migration and division). Albeit non-amenable to HCS screening, future MITOMATICS
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28 513 developments will use bioimaging techniques that could capture mitochondria in 4D, such as lattice
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30 514 light-sheet microscopy (LLSM) and three-dimensional structured illumination microscopy (3D SIM).
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32 515 For now, our method for 2D automated quantification of mitochondrial morphology appears to be well-
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34 516 suited for relatively ‘flat’ cells, i.e., cells where mitochondria are confined to a limited number of planes.
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36 517 Utilization of cells cultured in 3D (through different systems including spheroids, hydrogels or
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38 518 scaffolds) could improve physiological relevance for certain cell types (like endothelial cells or mixed
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40 519 cell populations such as in 3D reconstituted skin) or conditions (e.g. growth of cancer cells).
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46 520 5. Conclusions

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49 521 In vitro assessment of mitochondrial toxicity through our novel quantitative imaging system can be
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51 522 useful to draft prioritized lists of deleterious chemicals, ranked according to their impact on
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53 523 mitochondria, before functional tests and in vivo assays are conducted. Our HCS system may also serve
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55 524 as a valuable tool to transition from the current single-chemical-based risk paradigm towards one which
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57 525 addresses co-exposures to multiple chemicals, and more generally to multiple stressors due to external
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59 526 exposome and/or pathological conditions.
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3 528 6. Acknowledgements
4

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16 751 [Legends to Figures](#)

18 752

21 753 [Figure 1.](#)

24 754 **Overview of the MITOMATIC workflow.**

27 755 The main steps of automated analysis of mitochondrial morphology using the MITOMATIC workflow
28 are presented. Cellular models are selected (MITOGATE) to assess the mitochondria-disrupting effects
29 756 are presented. Cellular models are selected (MITOGATE) to assess the mitochondria-disrupting effects
30 of single chemicals (*MitoCollapse*) or of molecules used in combination (*MitoCocktail*). Fluorescent
31 757 of single chemicals (*MitoCollapse*) or of molecules used in combination (*MitoCocktail*). Fluorescent
32 images of live cultured cells with labeled mitochondria are captured and loaded into MitoRadar. The
33 758 images of live cultured cells with labeled mitochondria are captured and loaded into MitoRadar. The
34 images are segmented and a total of 104 morphological and texture features are extracted for each frame.
35 759 images are segmented and a total of 104 morphological and texture features are extracted for each frame.
36 The values can be plotted for a particular cell or for all cells present in a given microscopic field, at the
37 760 The values can be plotted for a particular cell or for all cells present in a given microscopic field, at the
38 level of the whole mitochondrial population or for mitochondrial subnetworks. The software handles
39 761 level of the whole mitochondrial population or for mitochondrial subnetworks. The software handles
40 data analysis, statistics and visual exploration for instance in the form of radar plots. Reference
41 762 data analysis, statistics and visual exploration for instance in the form of radar plots. Reference
42 condition is standardized by a blue circle for comparison with a different cellular state (lines with other
43 763 condition is standardized by a blue circle for comparison with a different cellular state (lines with other
44 colors). The basic steps of this wet-plus-dry pipeline are thus as follows: (i) Pre-acquisition steps: cell
45 764 colors). The basic steps of this wet-plus-dry pipeline are thus as follows: (i) Pre-acquisition steps: cell
46 treatment including culture in 96-well plates, drug exposure and staining. (ii) Acquisition step: live-cell
47 765 treatment including culture in 96-well plates, drug exposure and staining. (ii) Acquisition step: live-cell
48 imaging (5 random fields/well) acquired on an Opera Phenix High-Content Screening system (or other
49 766 imaging (5 random fields/well) acquired on an Opera Phenix High-Content Screening system (or other
50 imaging platform). (iii) Post-acquisition steps: fully automated image analysis by the MitoRadar
51 767 imaging platform). (iii) Post-acquisition steps: fully automated image analysis by the MitoRadar
52 software including image processing, AI-driven segmentation, parameter quantification, statistics,
53 768 software including image processing, AI-driven segmentation, parameter quantification, statistics,
54 visual representation and scoring. Note that the Graphical Abstract shows a differently annotated
55 769 visual representation and scoring. Note that the Graphical Abstract shows a differently annotated

770 version of the pipeline: the step of cellular model selection corresponds to ‘Question to protocol’, pre-
771 acquisition and acquisition steps are classed under the denomination ‘Cells to pixels’ and, lastly, post-
772 acquisition steps are referred to as ‘Pixels to Data’ and ‘Data to Answer’.

773

774 [Figure 2.](#)

775 **Implementation of the MITOPIX-DEV image dataset of BEAS-2B cells treated with**
776 **mitochondrial pro-fission and hyper-fusion drugs.**

777 BEAS-2B cells were treated with vehicle (0,5% DMSO), pro-fission drugs (20 μ M CCCP, 1 μ M
778 Rotenone and 1 μ M Antimycin A), and hyper-fusion drugs (50 μ M MDIVI-1 and 10 μ M M1) for 2h
779 and stained with HOE (blue), CMG dye (green) and MTDR (purple) before confocal imaging (on the
780 Opera Phenix HCS system). Images from the different fluorescent channels are presented separately or
781 merged (overlay). Zoom-ins are shown. Scale bar = 10 μ m.

782
783 [Figure 3.](#)

784 **Overview of the MitoRadar software.**

785 (A) MitoRadar main page. The left panel allows users to manage data and launch image processing
786 tasks while the center panel is dedicated to visualization (of both images and segmentation results). The
787 right panel shows the descriptor values and their distribution for a single field or on the whole dataset.
788 (B) MitoRadar basic features: a-Segmentation visualization panel (showing cells, nuclei, mitochondria
789 and skeletons); b-Experimental data import module to link experimental data from the plates to the
790 captured images; c-Advanced statistical panel to produce advanced statistical representations with PCA,
791 LDA, Correlation, T-Test, SSMD and MitoRadar plots; d-Example of a 3D LDA plot produced with
792 MitoRadar in-house statistical module. (C) The resulting data are plotted into MitoRadar charts, where
793 the reference condition is standardized by a blue circle for comparison with another experimental
794 condition of interest (orange line), such as chemical treatment or diseased status. Parameters can be

795 ranked in ascending order according to their SSMD values and they are colored in blue when the effect
796 is diminished, brown when it is low and red when it is increased. Significant differences from basal for
797 each experimental group are depicted by small (*: $p < 0.05$), medium (**: $p < 0.01$) or large (***:
798 $p < 0.001$) circles (Mann Whitney Test).

799

800 **Figure 4.**

801 **Quantitative analysis of mitochondrial morphology in BEAS-2B cells treated with the**
802 **mitochondrial uncoupler CCCP.**

803 (A) Representative confocal microscopy images of BEAS-2B cells treated with 0,5% DMSO (vehicle,
804 left panel) or 20 μ M CCCP (right panel) during 4h before mitochondrial staining (with MTRD). Scale
805 bar = 10 μ m. (B) More than 4,000 CCCP-treated cells were analyzed using the MitoRadar software; the
806 six different MitoRadar plots figure distribution of the normalized 104 morphometric features. The
807 upper three MitoRadar plots correspond to computation for total mitochondria (*Mito*), clustered
808 mitochondria (*Mitocluster*) or *Mitochondriome* (the full mitochondrial pool of a cell, considered as a
809 single object), whereas the bottom three MitoRadar plots result from calculations of *Nuclear*,
810 *Countershape* and cellular characteristics (*Cell*). Descriptors are colored in blue when the measured
811 effect is diminished and in red when it is increased. Significant differences from basal (blue circle) for
812 the CCCP-treated condition (orange line) are depicted by small (*: $p < 0.05$), medium (**: $p < 0.01$) or
813 large (***: $p < 0.001$) circles (Mann Whitney Test). Shown are representative data of $n=5$ independent
814 experiments. (C) MitoRadar can calculate a MitoScore based on the deviation and number of affected
815 parameters. Top 15 descriptors are listed. (D) Statistical analysis performed by MitoRadar. a- PCA plot
816 on the first two components, with arrows indicating descriptor correlation with each axis; b- Box plot
817 along the unique LDA axis; c- Violin plot showing a particular descriptor: Mito-roundness_mean, as
818 an example.

819

820 Figure 5.

821 **Quantitative analysis of mitochondrial morphology after acute exposure of human pulmonary**
822 **cells to single pesticides (*MitoCollapse*).**

823 (A) List of pesticides tested alone on BEAS-2B cells for 2h at different doses ranging from 10 to 500
824 μM , chemical category, threshold dose inferred by MitoRadar analysis and LD50 determined 24h post-
825 treatment. (B) BEAS-2B cells were treated for 2h with vehicle (0,5% DMSO), 20 μM of CCCP or
826 250 μM of individual pesticides (FEN, PYRI, MEP, THI, AZO or PYRA). MitoRadar plots (morpho-
827 phenotypic signatures) obtained after analysis by MitoRadar. For statistical analysis, a Mann-Whitney
828 U test was used. Each MitoRadar plot results from analysis of around 20,000 cells and shown data are
829 representative of 4 independent experiments. Note that when Mitoradar plots are computed with data
830 obtained 2h post-treatment with 100 μM of THI (**Fig. S6**, grey color), which is the calculated LD50/24h
831 value for this pesticide, changes in parameters indicative of mitochondrial toxicity are already visible
832 (with an increase in roundness_mean, circularity_mean, solidity_mean, compaction_mean and
833 concomitant decrease in area_mean, axis_major_length_mean, axis_minor_length_mean,
834 skel_length_mean). Complete single-compound dose-responses are shown in **Fig. S7**. (C) MitoScore
835 for estimating treatment effect. Shown are the most affected parameters along a color-coded scale with
836 explicit reference to one of the following five categories: *No effect*, *Limited* / *Substantial* / *Measurable*
837 or *Large overall effect*. (D) Bidimensional representation of the established group into the new subspace
838 generated by the first two LDA discriminant axis. Both LDA axis separate samples exposed to ETC
839 complex I, II and III inhibitors and CCCP from the untreated ones.

840

841 Figure 6.

842 **Quantitative analysis of mitochondrial morphology after acute exposure of human pulmonary**
843 **cells to pesticide mixtures (*MitoCocktail*)**

844 (A) List of pesticides tested alone on BEAS-2B cells for 4h at different doses ranging from 10 to 500
845 μM , chemical category, threshold dose inferred by MitoRadar analysis and LD50 determined 24h post-

1 846 treatment. Data shown are representative of at least 4 independent experiments with 4 replicates per
2 847 experiment. (B) Table listing the main air-borne pesticides found in the five departments of region
3
4 848 Occitanie (Southern France) and their relative percentage (according to real-world data produced by
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6 849 ATMO-Occitanie). The prominent pesticide (in red) detected in each of these five locations was tested
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8 850 at 5 doses ranging from 10 to 500 μM , the other pesticides in the mixture at the dose corresponding to
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10 851 their percentage in each region. The threshold dose inferred by MitoRadar and the LD50 are reported
11
12 852 in the third and fourth column of the Table. (C) Representative confocal microscopy images of
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14 853 mitochondria (stained with MTD) present in BEAS-2B cells after treatment with 0,5% DMSO (panel
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16 854 a), 10 μM FOL (panel b), 50 μM FOL (panel c) or with a mixture of pesticides found in the Aude
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18 855 department (one of the five departments of region Occitanie) with main pesticide (FOL) concentration
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20 856 set at 10 μM (panel d). (D) MitoRadar plots showing variations in mitochondrial parameters after
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22 857 treatment with vehicle (0,5% DMSO, blue circle), 10 μM (orange), 50 μM (green) or 100 μM (red) of
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24 858 FOL alone (left MitoRadar) or by the mixture of pesticides found in Aude with the main pesticide (FOL)
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26 859 concentration set at 10, 50 or 100 μM (respectively in orange, green and red in the right panel). (E)
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28 860 MitoScore for estimating treatment effect. Shown are the most affected parameters along a color-coded
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30 861 scale with explicit reference to one of the following five categories: *No effect*, *Limited* / *Substantial* /
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32 862 *Measurable* or *Large overall effect*.

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39 864 [Supplementary Information](#)

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42 865 [Supplementary Figure 1 \(Fig. S1\)](#).

43 866 **MitoRadar segmentation module.**

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50 867 (A) Labelled nuclei, membranes and mitochondria are imaged and pictures are loaded into the
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52 868 MitoRadar software. The images are processed through successive segmentation, labelling and
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54 869 transformation steps. For the first segmentation step which consist in detecting cells and nuclei,
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56 870 MitoRadar uses deep learning. Image processing and morphometric measurements are fully automated
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58 871 and can be parallelized. (B) Descriptors pertaining to multiscale (wells /fields /cells / nuclei /
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1 872 mitochondriome / countershape /clusters of mitochondria/ isolated mitochondria) and multidimensional
2 873 analysis (n=104) for identification of complex phenotypes. (C) Segmentation step (detail). Tri-channel
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4 874 staining (a): cell membranes are labeled in green, nuclei in blue and mitochondria in purple. Cell label
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6 875 (b). Nuclei label (c). Clustered mitochondria label (d). Mitochondrial skeleton label (e). Isolated
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8 876 mitochondria label (f). Note that in our image analysis workflow, images were “down-sampled” only
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10
11 877 for the nuclei and cytoplasm channels to maximize segmentation efficacy and computational efficiency
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13 878 (these objects being large enough, this operation only minimally affects their definition). Mitochondrial
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15 879 segmentation was carried out at the maximum image resolution (i.e., images were processed at full
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17 880 resolution without any pre-processing step).

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26 883 [Supplementary Table 1 \(Table S1\).](#)

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29 884 List of descriptors, classification and definition.

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35 886 [Supplementary Figure 2 \(Fig. S2\).](#)

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38 887 **Fluorescence imaging of eight human cell lines.**

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40 888 Representative confocal microscopy images of the following cell lines: BEAS-2B (non-tumorigenic
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42 889 lung epithelial cells), A549 (lung adenocarcinoma cells), RPE-1 (retinal pigment epithelial-1 cells),
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44 890 U2OS (osteosarcoma cells), Hs68 (foreskin fibroblasts), THLE-3 (liver epithelial cells), MDA-MB231
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46 891 (triple-negative breast cancer cells) and HK-2 (kidney proximal tubule cells). Nuclei were labelled using
47
48 892 Hoechst (blue; HOE), membranes with CellMask dye (green; CMG) and mitochondria with the
49
50 893 mitochondria-specific dye MitoTracker Deep Red FM (purple; MTDR). Pictures were taken by live-
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52 894 cell confocal microscopy on the Opera Phenix[®] HCS system. Images from the different fluorescent
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54 895 channels are presented separately or merged (overlay). N=2 independent experiment with 10 replicates
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56 896 for each condition and more than 10,000 analyzed fields. Zoom-ins are shown. Scale bar = 10µm.

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2
3 898 Supplementary Figure 3 (Fig. S3).

4
5 899 **Treatment of different human cell lines with mitochondrial pro-fission and hyper-fusion drugs**
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8 900 **for implementation of the MITOPIX-DEV image dataset.**

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10 901 BEAS-2B, A549, RPE-1, U2OS, Hs68, THLE-3, MDA-MB231 and HK-2 cells were treated with
11
12 902 vehicle (DMSO), pro-fission drugs (20 μ M CCCP, 1 μ M Rotenone and 1 μ M Antimycin A), and hyper-
13
14
15 903 fusion drugs (50 μ M MDIVI-1 and 10 μ M M1) for 2h and stained with HOE (blue), CMG dye and
16
17 904 MTDR before confocal imaging (on the Opera Phenix[®] HCS System). Images from the different
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19 905 fluorescent channels were merged (overlay). Zoom-ins are presented. Scale bar = 10 μ m.

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25 907 Supplementary Figure 4 (Fig. S4)

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27
28 908 **Overview of MitoRadar statistical analysis system (example of CCCP treatment).**

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30
31 909 (A) Summary of the SSMD based on comparison between control cells (0.5% DMSO, vehicle) and
32
33 910 cells treated with a pro-fission drug (20 μ M CCCP for 4h). This measure of the size effect is greater
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35 911 between both groups when the square is red, and smaller when the square is blue. (B) and (C) U- and
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37 912 p-value computations using Mann-Whitney test and T-test respectively, plus Benjamini-Hochberg
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39 913 correction. When the difference between the compared groups is statistically significant for a given
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41 914 variable, the squares are labeled in dark green (see for instance Mito_roundness_mean), whereas when
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43 915 the differences are not significant, the squares are labeled in grey (see for instance the whole set of
44
45 916 nuclear parameters). (D) Hierarchical clustering of the computed parameters. (E) Correlation distance
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47 917 map showing the dependence between descriptors. Detailed view of the correlation of descriptors along
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49 918 each axis for PCA (F) and LDA (G).

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57 920 Supplementary Figure 5 (Fig. S5)

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60 921 **MitoRadar analytical platform with advanced statistics.**

922 (A) MitoRadar plots showing features associated with nuclei, countershape and cells, after 2h exposure
923 to 250µM of pesticides (FEN in red, PYRI in pink, MEP in violet, THI in grey, AZO in orange and
924 PYRA in brown), 20 µM CCCP (in green) or 0,5% DMSO (blue circle as a reference). (B) Correlation
925 distance map showing dependence between descriptors. (C) SSMD summary showing size effect. (D)
926 U-values computations using Mann-Whitney test and significance level after Benjamini-Hochberg
927 correction.

928

929 [Supplementary Figure 6 \(Fig. S6\)](#)

930 **Quantitative analysis of mitochondrial morphology after acute exposure of human pulmonary**
931 **cells to 100µM of single pesticides.**

932 BEAS-2B cells were treated for 2h with vehicle (0,5% DMSO), 20µM of CCCP or 100µM of
933 individual pesticides (FEN, PYRI, MEP, THI, AZO or PYRA). MitoRadar plots (morpho-
934 phenotypic signatures) obtained after analysis by MitoRadar. For statistical analysis, a Mann-
935 Whitney U-test was used. Each MitoRadar plot results from analysis of around 20,000 cells
936 and shown data are representative of 4 independent experiments.

937

938 [Supplementary Figure 7 \(Fig. S7\)](#)

939 **Quantitative analysis of mitochondrial morphology after acute exposure of human pulmonary**
940 **cells to 10-500µM of single pesticides (dose-reponses).**

941 BEAS-2B cells were treated for 2h with vehicle (0,5% DMSO, blue circle), 20µM of CCCP (pink
942 broken line) or with 10µM (orange), 50 µM (purple), 100µM (green), 250µM (red), or 500µM (brown)
943 of individual pesticides (FEN, PYRI, MEP, THI, AZO or PYRA). MitoRadar plots (morpho-phenotypic
944 signatures) obtained after analysis by MitoRadar. For statistical analysis, a Mann-Whitney U-test was
945 used. Each MitoRadar plot results from analysis of around 20,000 cells and shown data are
946 representative of 4 independent experiments.

Figure 1



[Click here to access/download;Figure;Fig1.pdf](#)

MITO GATE

SELECT CELLULAR MODELS



MITO COLLAPSE
PREDICTIVE TOXICOLOGY

MITO COCKTAIL
ECOTOXICOLOGY

The experimental workflow includes:

- Cell culture plates with red media.
- Microcentrifuge tubes containing reagents.
- Fluorescence microscopy images of cells stained for mitochondria (green) and nuclei (blue).
- The MITO RADAR software interface, showing:
 - Fields:** ID, Field metadata (Project, Predictive Single, Combination, Date, Well_status, Tissue_region, Cell_health_status, Cell_line, BEAS-25, Patient_ID, Organism, human, Reagent_name, DMSO, Reagent_dose, 000, 100%, Reagent_duration, 0h).
 - Dataset metadata:** Plate, Date, Well, Well_status.
 - Cell:** Cell_health_status, p13, p53, p8.
 - Reagents:** OCCP, DMSO.
 - Fast Descriptors:** Cell, Nucleus, Mitochondrion, Countershape, Cluster count, Area, Mitochondria, Mito Cluster.
 - Fast Images:** Dataset, Duration.
 - Image Processing:** Segmentation, Use GPU, Quantification, Use GPU.
 - Filter:** Exclude Selected, Keep Only Selected, Repeat Filter, Rename Selected, Sort Selection Numerically.
 - Legend:** Mito, Cell, Nucleus, Mito label, Cell label, Nucleus label, Cluster label, Mito label, Skeleton.
- 3D scatter plot of data points.
- 2D circular radar chart showing parameter distributions.
- Color scale legend for the radar chart.

Figure 2

DMSO

CCCP

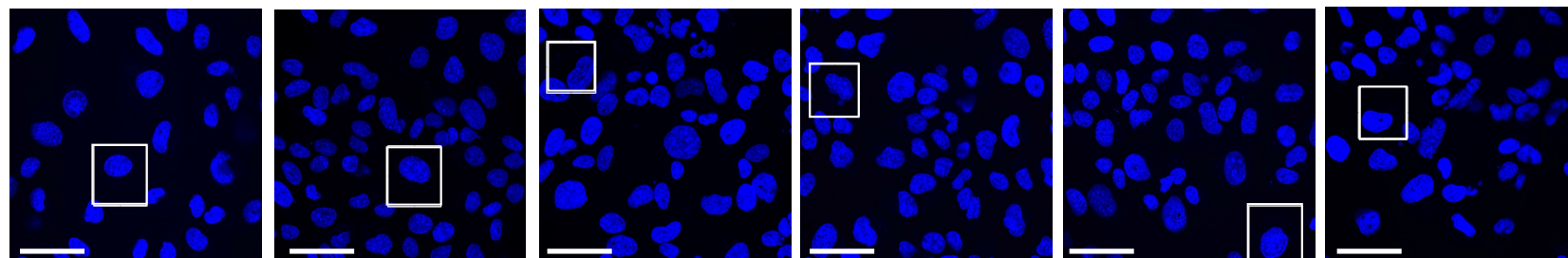
Rot

Ant A

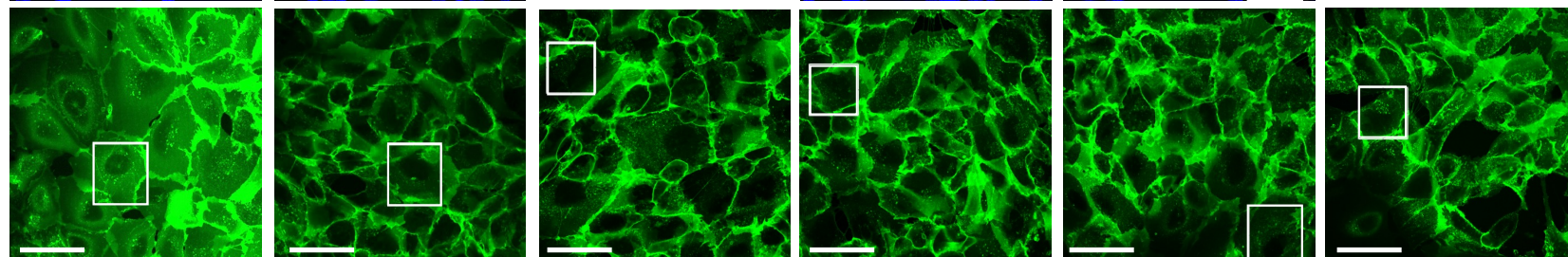
MDIVI-1

M1

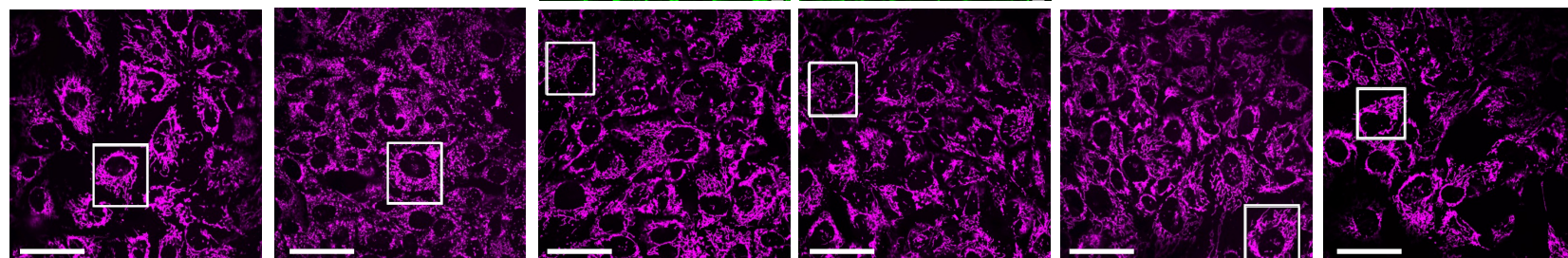
HOE



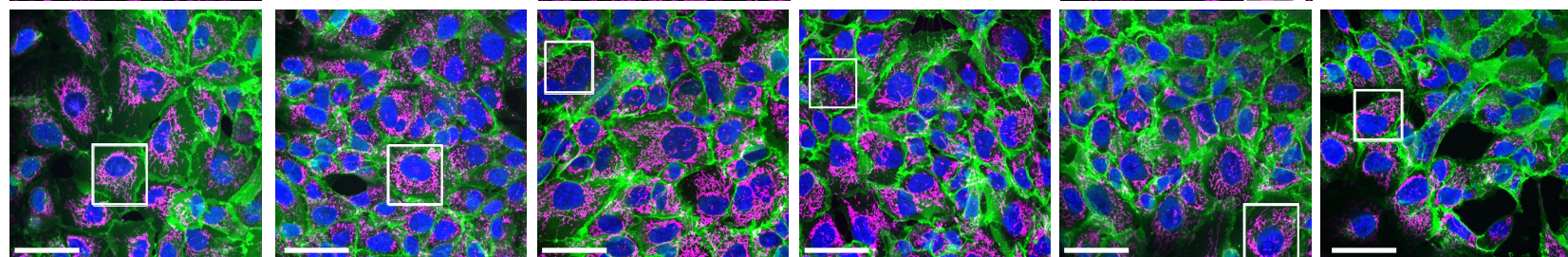
CMG



MTDR



Merge



Zoom

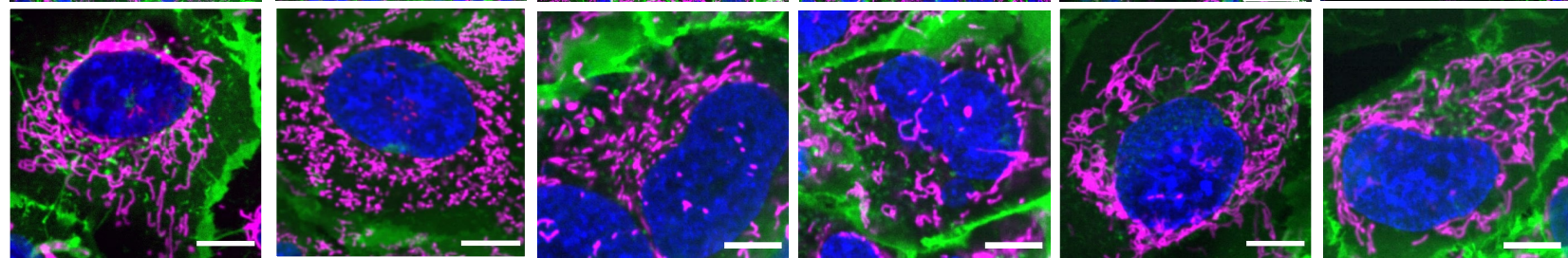
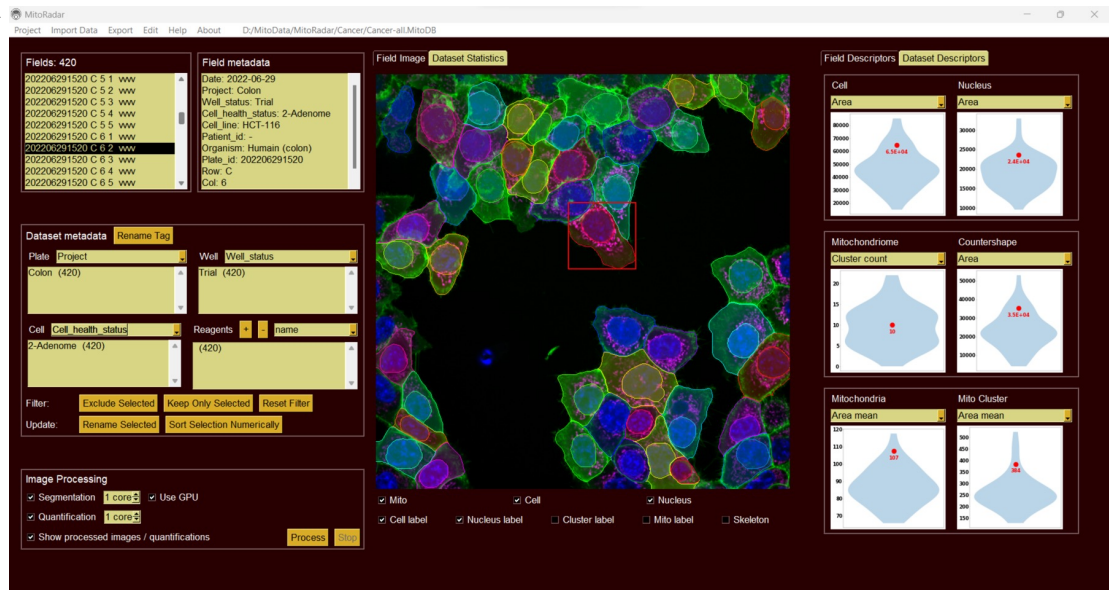
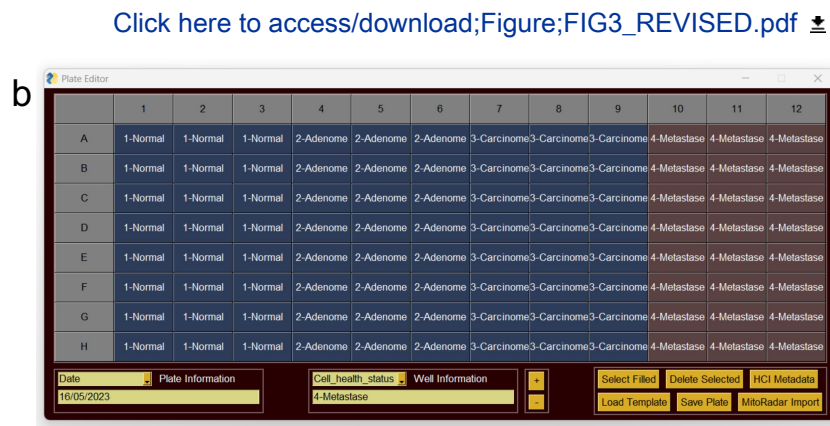
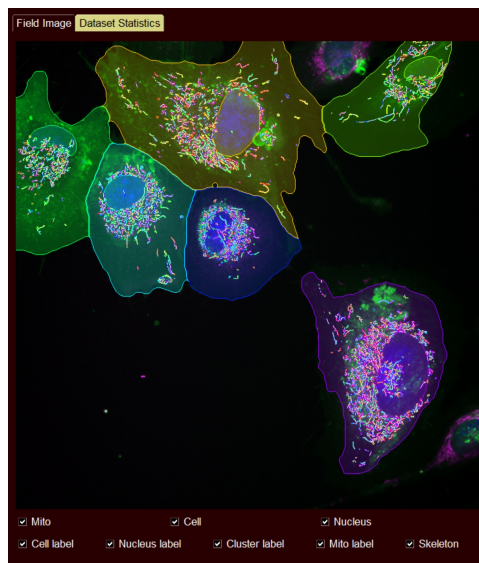


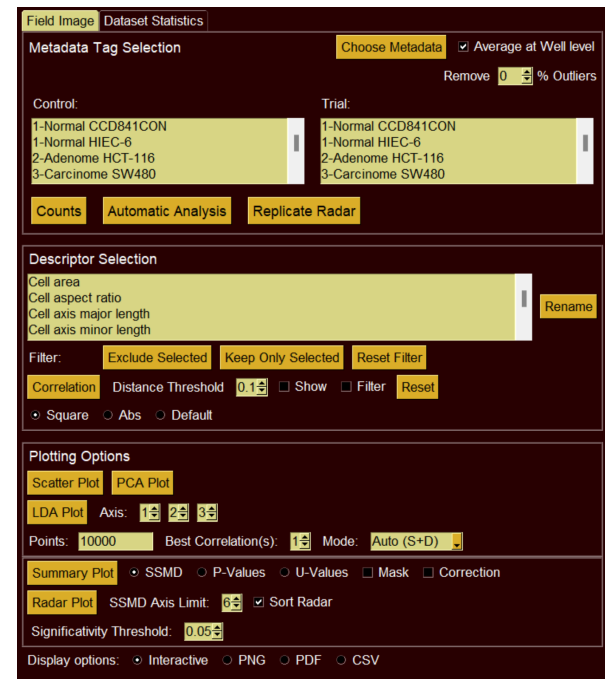
Figure 3



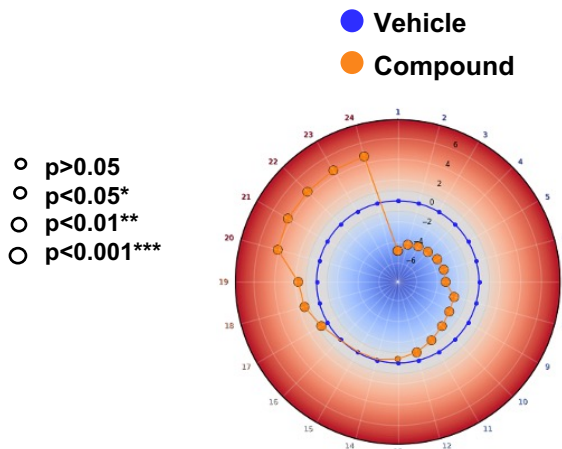
B



C



C



- MITOCHONDRIA**
- curl_mean
 - aspect_ratio_mean
 - skel_length_mean
 - eccentricity_mean
 - perimeter_crofton_mean
 - axis_major_length_mean
 - feret_diameter_max_mean
 - area_mean
 - relative_position_mean
 - equivalent_diameter_area_mean
 - axis_minor_length_mean
 - relative_orientation_mean
 - intensity_min_mean
 - dist_to_nucleus_mean
 - intensity_mean_mean
 - intensity_max_mean
 - orientation_mean
 - euler_number_mean
 - dist_to_membrane_mean
 - compaction_mean
 - circularity_mean
 - roundness_mean
 - extent_mean
 - solidity_mean
- Diminishing (blue) to Low Increasing (red)

d

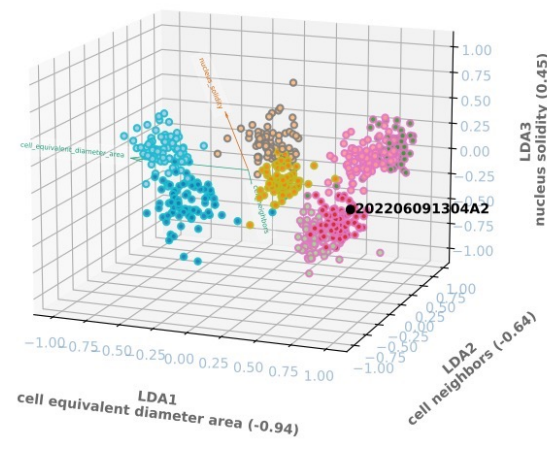
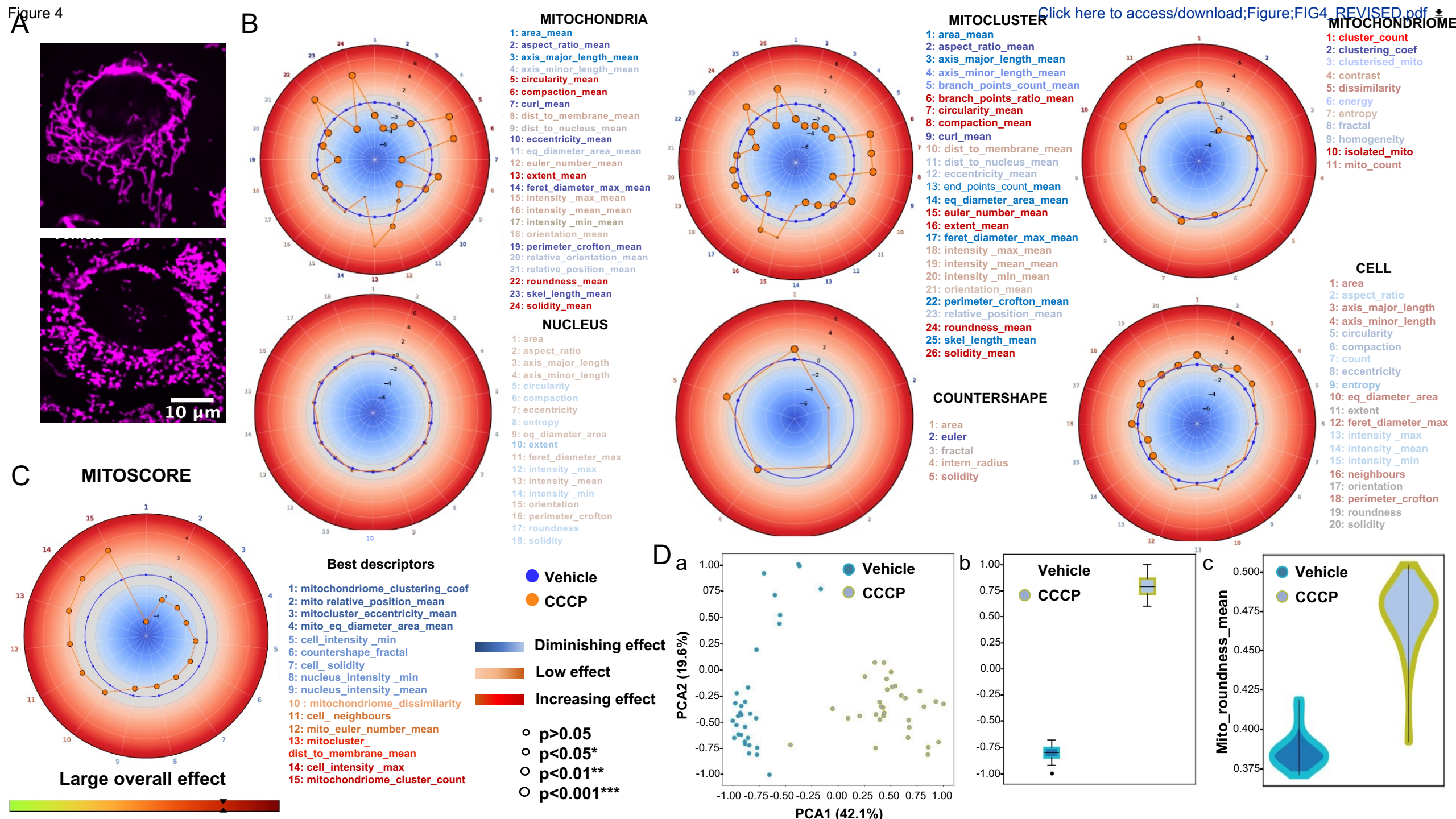


Figure 4



A

PESTICIDES	CATEGORY	THRESHOLD DOSE	LD50
FENPYROXIMATE	ACARICIDE	50µM	>500µM
PYRIDABEN	ACARICIDE	50µM	>500µM
MEPRONIL	FONGICIDE	50µM	≥ 500µM
THIFLUZAMIDE	FONGICIDE	10µM	100µM
AZOXYSTROBIN	FONGICIDE	100µM	≥ 500µM
PYRACLOSTROBIN	FONGICIDE	10µM	>500µM

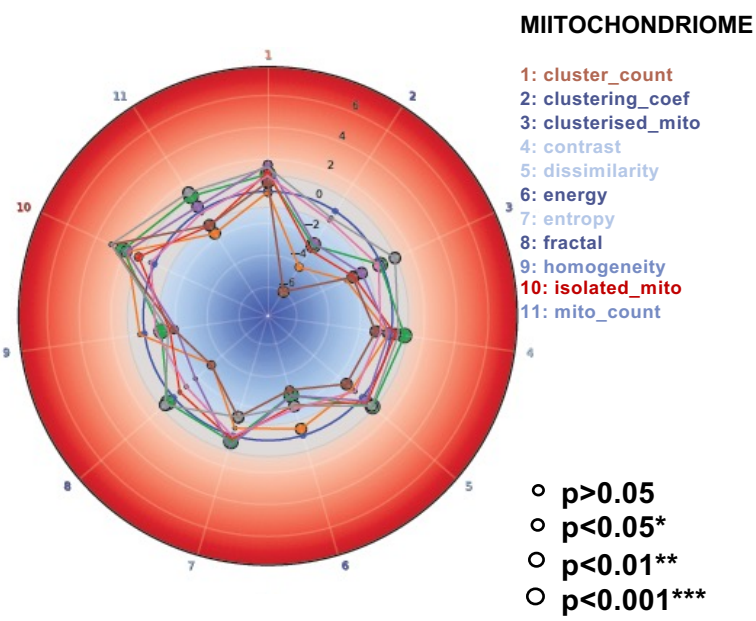
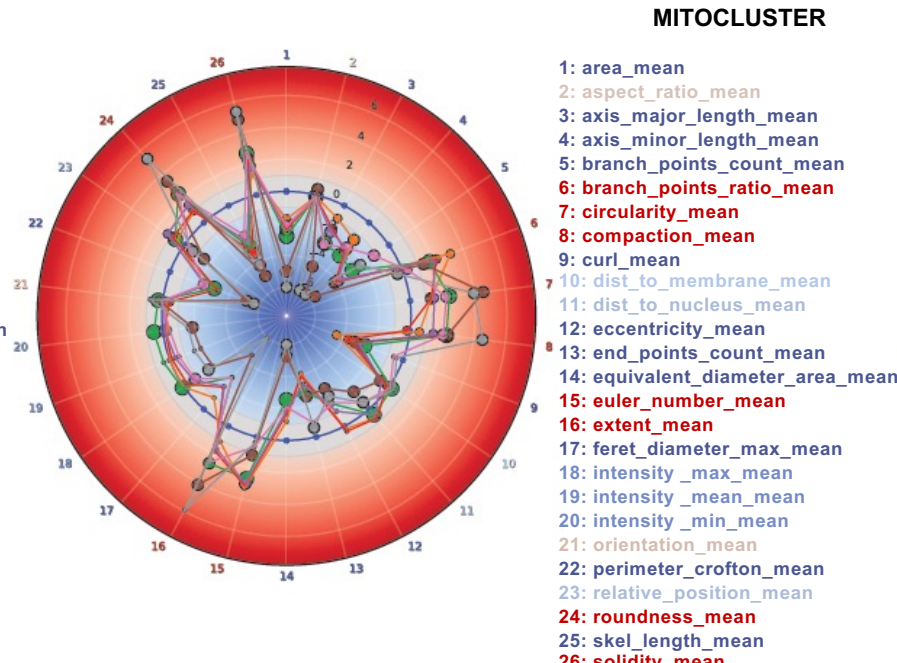
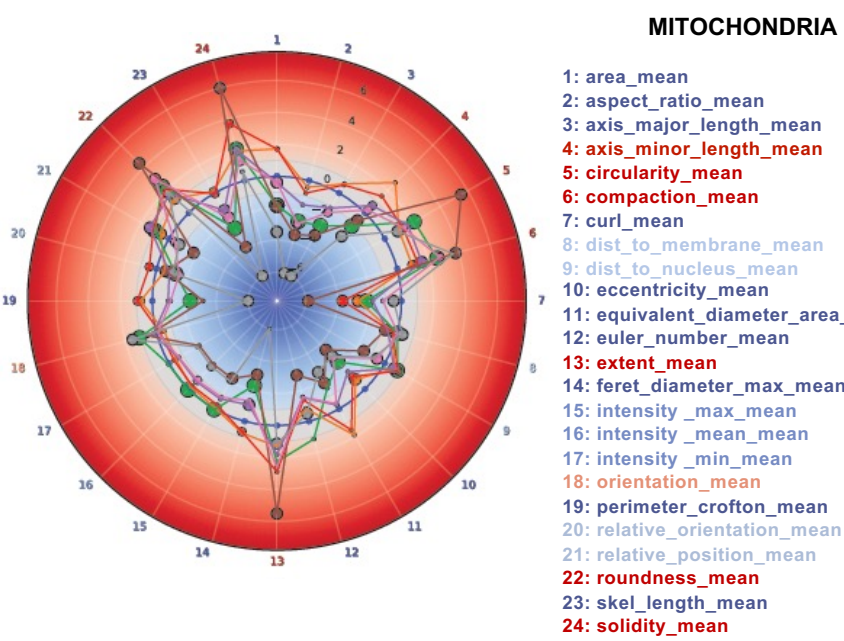
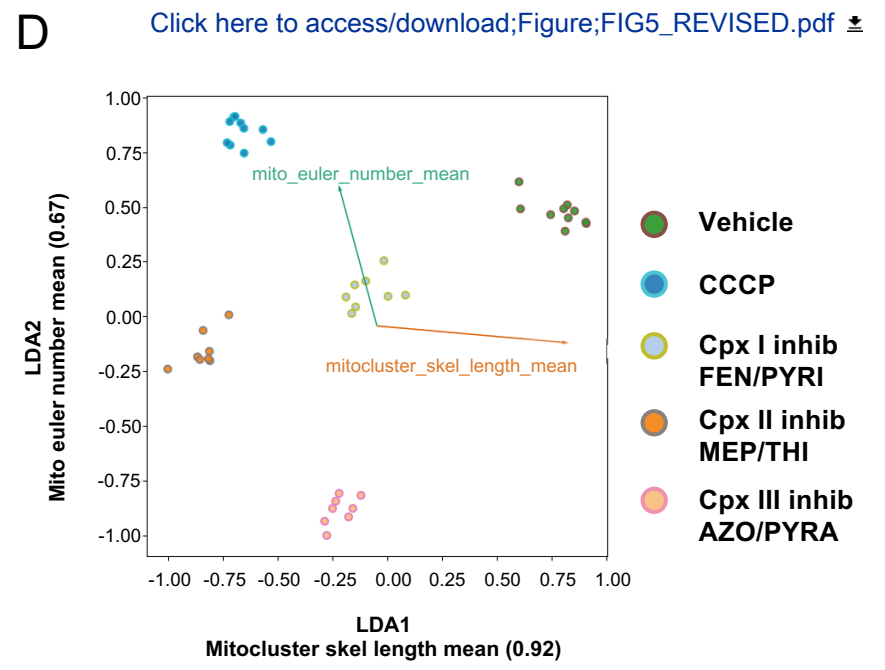
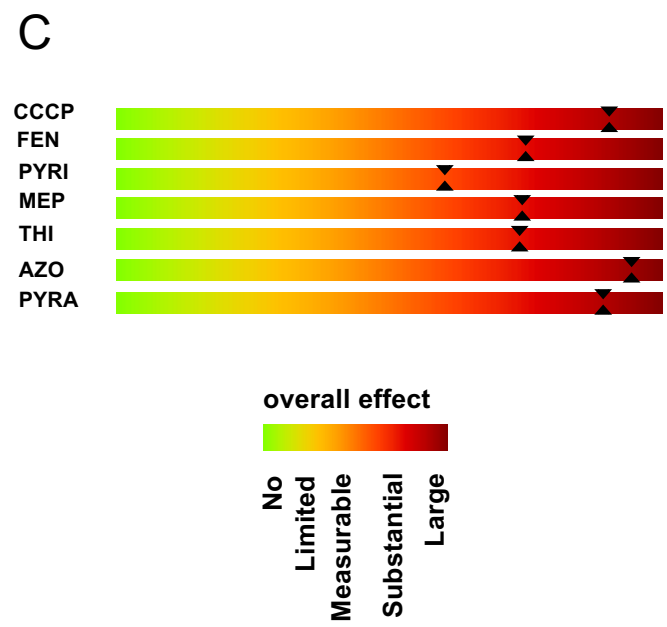


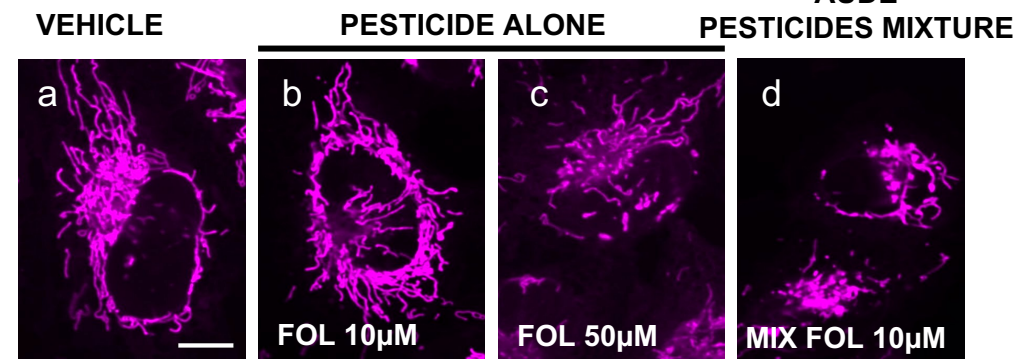
Figure 6

PESTICIDES	CATEGORY	THRESHOLD DOSE	LD50
FOLPEL	FONGICIDE	50 μ M	150 μ M
PENDIMATHALINE	HERBICIDE	250 μ M	>500 μ M
CHLORPYRIPHOS	FONGICIDE	50 μ M	100 μ M
LINDANE	INSECTICIDE	500 μ M	500 μ M
CYPRODINIL	FONGICIDE	100 μ M	75 μ M

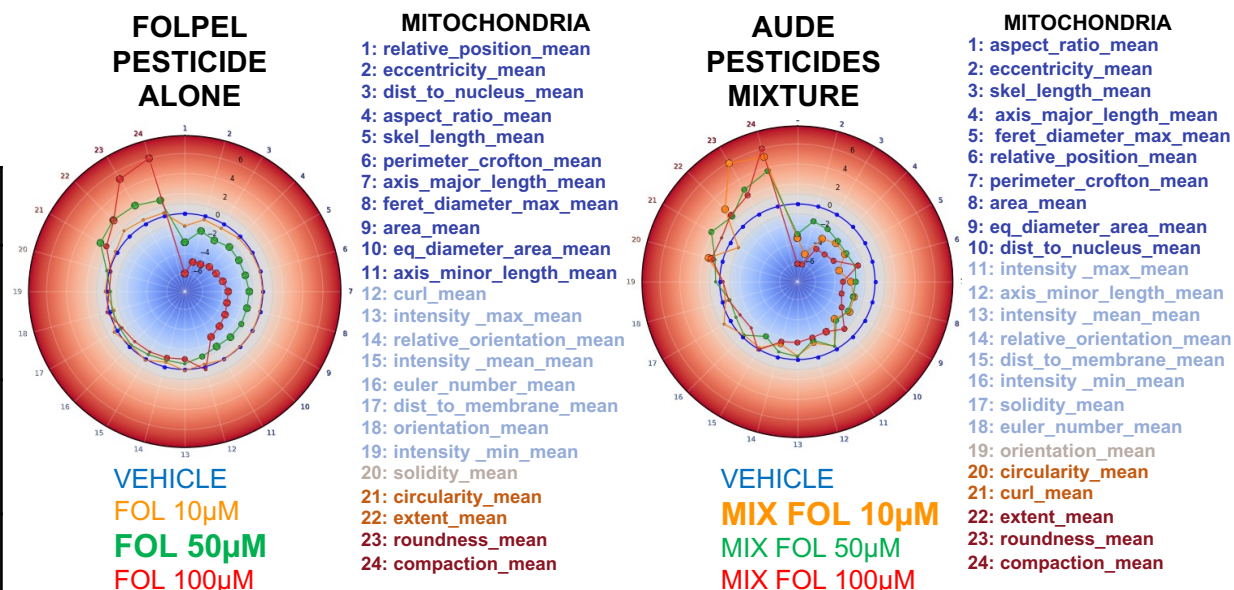
B

DEPARTMENT	PESTICIDE COMBINATION	PERCENTAGE IN MIXTURE	THRESHOLD DOSE	LD50
TARN ET GARONNE	PENDIMATHALINE FOLPEL LINDANE CYPRODINIL	83% 8% 6,5% 2,2%	50μM 4,8 μ M 1,3 μ M 3,9 μ M	100μM 9,6 μ M 2,6 μ M 7,8 μ M
PYRENEES-ORIENTALES	FOLPEL PENDIMATHALINE CHLORPYRIPHOS CYPRODINIL	88% 4% 4% 4%	10μM 0,47 μ M 0,47 μ M 0,47 μ M	10μM 0,47 μ M 0,47 μ M 0,47 μ M
LAURAGAIS	PENDIMATHALINE FOLPEL LINDANE	78% 20% 1,8%	50μM 13,3 μ M 1,2 μ M	100μM 27 μ M 2,4 μ M
AUDE	FOLPEL PENDIMATHALINE CHLORPYRIPHOS LINDANE	97% 1,1% 0,8% 1,5%	10μM 0,11 μ M 0,15 μ M 0,08 μ M	10μM 0,11 μ M 0,15 μ M 0,08 μ M
GARD	FOLPEL PENDIMATHALINE CHLORPYRIPHOS	93% 1,5% 5%	10μM 0,2 μ M 0,54 μ M	10μM 0,2 μ M 0,54 μ M

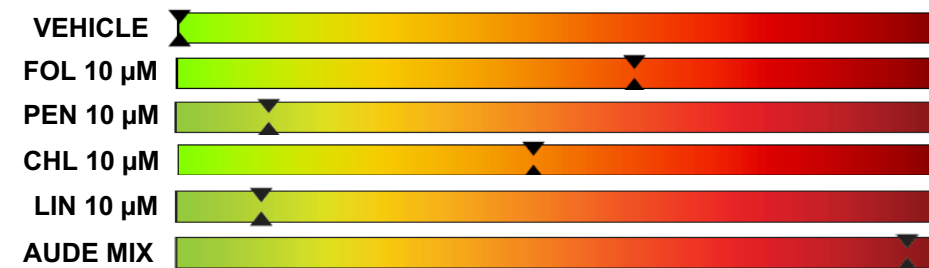
C



D



E



Declarations of interest: none.

Sophie Charrasse: Data curation; formal analysis; investigation; visualization; methodology; writing – review and editing.

Charlotte Saint-Omer: Data curation; formal analysis; investigation; visualization; methodology.

Benoît Bordignon: Data curation; formal analysis; investigation; visualization; methodology.

Manuela Pastore : Data curation; software; formal analysis; investigation; visualization; methodology; writing – review and editing.

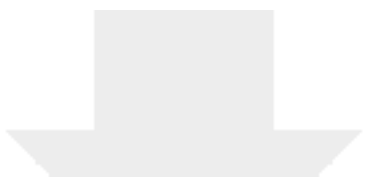
Richard E. Frye : Resources; data curation; methodology; writing – review and editing.

Titouan Poquillon : Data curation; software; formal analysis; investigation; visualization; methodology; writing – review and editing.

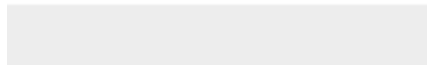

Victor Racine : Data curation; software; formal analysis; investigation; visualization; methodology; writing – review and editing.


Christelle Reynes : Data curation; software; formal analysis; investigation; visualization; methodology; writing – review and editing.

Abdel Aouacheria: Conceptualization; data curation; formal analysis; supervision; funding acquisition; investigation; visualization; writing – original draft; project administration; writing – review and editing.



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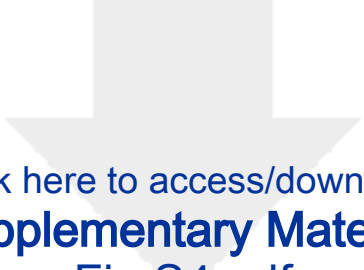


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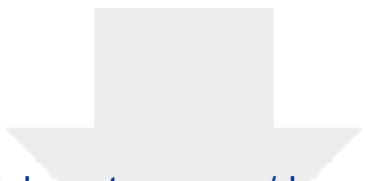


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